# Proteins in Frozen Solutions: Evidence of Ice-Induced Partial Unfolding

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ABSTRACT From a drastic decrease in the phosphorescence lifetime of tryptophan residues buried in compact rigid cores of globular proteins, it was possible to demonstrate that freezing of aqueous solutions is invariably accompanied by a marked loosening of the native fold, an alteration that entails considerable loss of secondary and tertiary structure. The phenomenon is largely reversible on ice melting although, in some cases, a small fraction of macromolecules recovers neither the initial phosphorescence properties nor the catalytic activity. The variation in the lifetime parameter was found to be a smooth function of the residual volume of liquid water in equilibrium with ice and to depend on the morphology of ice. The addition of cryoprotectants such as glycerol and sucrose profoundly attenuates or even eliminates the perturbation. These results are interpreted in terms of adsorption of protein molecules onto the surface of ice.

## INTRODUCTION

The solidification of water has dramatic consequences for many living organisms. Among the causes of freeze injury and death in living organisms is the cold lability of protein molecules (Franks, 1985). In vitro, freezing may result in irreversible protein aggregation and severe loss of catalytic function, reasons for which many proteins cannot be stored in ice or lyophilized from it without partial inhibition of their activity (Franks, 1985). In spite of the relevance of the phenomenon, little is known of the structure of proteins in ice or of the nature of the underlying perturbation. To date, the hypotheses advanced on the freeze damage mechanism are based on indirect evidence and are mainly construed around the presumed action of cryoprotectants, compounds that at relatively high concentrations prevent these alterations. The paucity of structural information on proteins in the frozen state is due primarily to the insensitivity or the poor resolution of ordinary spectroscopic methods, or both, in such a highly scattering and anisotropic medium as ice. We have overcome these limitations by employing the phosphorescence emission of tryptophan (Trp) residues as a monitor of the polypeptide dynamical structure; this is an intrinsic probe that has been instrumental in disclosing conformational changes of proteins in response to variations in conditions of the medium (Strambini and Gabellieri, 1984; Strambini and Gonnelli, 1988; Cioni and Strambini, 1994) or elicited by the binding of substrate and effector molecules (Galley and Strambini, 1976; Cioni and Strambini, 1989; Strambini and Gonnelli, 1990; Strambini et al., 1992). The proteins selected for this study, monomers, dimers, and tetramers, have known crystallographic structure and possess a sole Trp residue per subunit that exhibits a long-lived, room-temperature phosphorescence emission. Given the direct correlation between the phosphorescence lifetime $\tau$ and the rigidity of the protein matrix surrounding the chromophoric probe (Strambini and Gonnelli, 1985), the choice of long-lived Trp residues permits us to focus our attention specifically on well-structured, compact cores of the macromolecules.

## MATERIALS AND METHODS

Liver alcohol dehydrogenase from horse (LADH) was obtained as a crystalline suspension from Boehringer (Mannheim, Germany). Electrophoretically purified alkaline phosphatase (AP) type III-R from *Escherichia coli*, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) from yeast, and lactic dehydrogenase (LDH) from rabbit muscle were obtained from Sigma Chemical Co. (St. Louis, MO). Lys25-ribonuclease $T_1$ (RNase $T_1$) was purchased from Calbiochem Corp. (San Diego, CA). Apoazurin, obtained by removing copper from holoazurin (*Pseudomonas aeruginosa*), was a gift of Dr. N. Rosato, Department of Biochemistry, University of Tor Vergata, (Rome, Italy). Spectroscopic-grade ethylene glycol and glycerol, from Merck (Darmstadt, Germany), and sucrose, from Bio-Rad Laboratories (Richmond, CA), were used without further purification. Water, doubly distilled over quartz, was further purified by a millipore system.

All proteins were dialyzed overnight under nitrogen against a 1-mM K-phosphate buffer, pH 7, except for RNase $T_1$ for which the pH was 5. Any remaining insoluble precipitate was removed by centrifugation. Fresh preparations were made weekly, and for the enzymes no loss of activity was found during that time.

Luminescence experiments were carried out at a protein concentration of 3–5 $\mu$M, unless otherwise specified. The samples were placed in cylindrical spectrosil cuvettes of 4-mm i.d. Before phosphorescence measurements were made, oxygen was completely removed from the sample by a procedure described elsewhere (Strambini and Gonnelli, 1990). At any given temperature, we prepared partly frozen solutions by first seeding the supercooled liquid at -2°C with a small ice crystal and then bringing the sample to the desired subfreezing temperature in 2–3 min. After an equilibration time of 15 min, the results were largely independent of the direction, toward low or high temperature, chosen for obtaining lifetime-versus-temperature profiles.

Routine fluorescence spectra were obtained with a Jasco model FP-770 spectrofluorometer. A conventional homemade instrument was employed for all phosphorescence intensity and spectra measurements (Strambini and Gonnelli, 1990). The excitation provided by a Cermax xenon lamp

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Abbreviations used: $\tau$, Trp phosphorescence lifetime; LADH, alcohol dehydrogenase from horse liver; AP, alkaline phosphatase from *E. coli*; GAPDH, glyceraldehyde-3-phosphate dehydrogenase from yeast; LDH, lactic dehydrogenase from rabbit muscle.

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**TABLE 1 Change in Trp phosphorescence lifetime and intensity of proteins induced by freezing of aqueous solution at −10°C**

| Protein | Phosphorescent Trp | $\tau_0$ (s)* | $\tau_{ice}/\tau_0$ | $P_{rel}$‡ | $\tau_{rel}$‡ |
|---|---|---|---|---|---|
| *Monomers* | | | | | |
| Azurin | 48 | 1.950 | 0.03 | 0.73 | 0.98 |
| Ribonuclease $T_1$ | 59 | 0.130 | 0.10 | 0.97 | 0.93 |
| *Dimers* | | | | | |
| LADH | 314 | 1.450 | 0.035 | 0.87 | 0.90 |
| AP | 109 | 3.650 | 0.25 | 0.95 | 0.97 |
| *Tetramers* | | | | | |
| GAPDH | 84 | 0.370 | <0.01 | 0.59 | 0.67 |
| LDH | ? | 0.077 | 0.44 | 0.47§ | 1.00 |

*$\tau_0$ is the average phosphorescence lifetime in supercooled solutions.
‡$P_{rel}$ and $\tau_{rel}$ refer, respectively, to the relative phosphorescence intensity and lifetime in supercooled solutions after 10 freeze–thaw cycles. Drops in $P_{rel}$ occur when $\tau$ is shorter than 2 ms, the detection limit of the apparatus. $P_{rel}$ provides a measure of the fraction of proteins that have not undergone the dramatic shortening of $\tau$ commonly associated with extensive unfolding or denaturation.
§LDH samples were partly precipitated after thawing.

(LX 150UV, ILC Technology, Sunnyvale, CA) was selected by a 250-mm grating monochromator (H25, Jobin-Yvon, Longjumeau, France), and the emission was detected with an EMI 9635 QB photomultiplier. Phosphorescence decays were obtained following pulsed excitation by a frequency-doubled flash-pumped dye laser (UV 500 M-Candela) with a pulse duration of 1 μs and an energy per pulse typically of 1–10 mJ. The decay of Trp phosphorescence was monitored at 430 nm by an electronic shutter arrangement that permitted the emission to be detected 2 ms after the excitation pulse. The decaying signal was digitized and averaged by a computerscope system (EGAA; RC Electronics, Santa Barbara, CA). Subsequent analysis of decay curves in terms of a sum of discrete exponential components or in terms of a continuous Gaussian distribution of lifetime components was carried out by a nonlinear least-squares fitting algorithm, implemented by the program Global Analysis (Global Unlimited, LFD University of Illinois, Urbana). All decay data reported here are averages obtained from three or more independent measurements. Compared with the exponential behavior in liquid solutions, in partly frozen solutions the phosphorescence decay is always nonexponential in time. The average lifetime is $\tau = \Sigma\alpha_i\tau_i$, where $\alpha_i$ and $\tau_i$ are, respectively, the amplitude and the lifetime of the $i$th component obtained by analysis of the luminescence decay in terms of a sum of three exponential functions. The reproducibility of $\tau$ was typically better than 6% and never exceeded 10%.

## Results and Discussion

### Effect of ice formation on the Trp phosphorescence lifetime of proteins

The six proteins examined and the Trp residues responsible for the phosphorescence signal are listed in Table 1. Unlike the luminescence spectra, $\tau$ is markedly affected by the solidification of water. It is instructive first to consider how $\tau$ relates to temperature-induced variations of protein flexibility in solutions that do not undergo phase transitions. A typical example of a $\tau$-versus-temperature profile is given in Fig. 1, where the decay kinetics of azurin in a nonfreezing solvent (ethylene glycol/buffer, 50/50, w/w) is compared with that in buffer. At 170 K the organic solvent mixture forms a rigid glass; protein motions are blocked by the high viscosity, and $\tau$ assumes its upper limiting value of 6 s. When the solutionis warmed, $\tau$ decreases, first gradually, until on approaching the temperature of thermal denaturation it suddenly drops to below the 0.01-ms detection limit. Whereas the gradual reduction of $\tau$ reflects a progressive gain in the macromolecule flexibility as a result of the decreased solvent viscosity/increased thermal energy, the steep high-temperature drop (not shown in the figure) signals the abrupt enhancement of segmental mobility that accompanies the unfolding of the globular structure. In contrast to that of the organic solvent mixture, the $\tau$-versus-

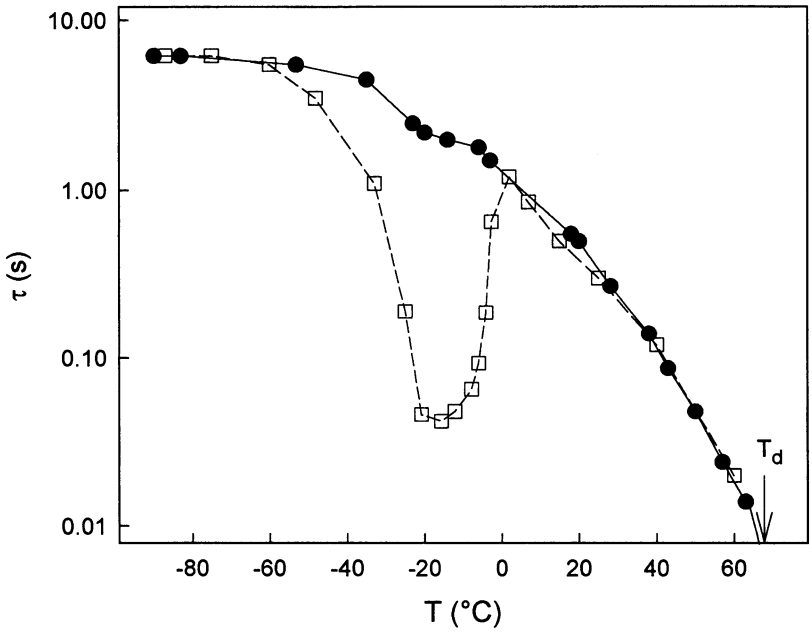


FIGURE 1 Dependence of the average phosphorescence lifetime $\tau$ of Trp-48 of azurin on temperature and solvent composition: ([●) ethylene glycol/K-phosphate buffer (1 mM, pH 7), 50/50 (w/w), and (□) K-phosphate buffer alone. $T_d$ is the temperature of thermal denaturation.

temperature profile in buffer exhibits an additional and sudden downward transition in correspondence of ice formation. For azurin at -12°C, as the supercooled solution solidifies, $\tau$ drops by ~30 folds, indicating a dramatic gain in the polypeptide flexibility. The phenomenon was observed with all six proteins examined (Table 1), and because it is independent of the number of subunits in the macromolecule the loosening of the structure cannot, in general, be ascribed to the dissociation of the subunits. These findings are the more surprising if one recalls that the solidification of water enhances the viscosity of the unfrozen liquid. From the coupling of protein motions to solvent viscosity (Gavish, 1980; Gonnelli and Strambini, 1993), a drastic increase in protein flexibility on freezing is thus totally unwarranted. We must conclude that the ice-induced decrease of $\tau$ attests to a general and extended loosening of the native fold, a partial unfolding that involves even the rigid protein domains hosting the phosphorescence probe.

It should be mentioned that the reduction of $\tau$ on freezing could, in principle, have a more trivial explanation, namely, enhanced quenching of Trp phosphorescence by trace impurities that, like all solutes, become highly concentrated in the unfrozen liquid. However, multiple observations prove that the influence of diffusion-mediated quenching reactions is negligible in ice, where the viscosity is relatively large. For example, the addition of oxygen, a most efficient quencher of protein phosphorescence, such as to reduce $\tau$ in the liquid phase by a factor of 2–3, did not affect the decay rate in ice. Further, as will be shown below, the actual drop in $\tau$ is influenced by the morphology of ice and the nature of solutes added to the starting solution.

Besides the drastic reduction in $\tau$, another salient feature of the phosphorescence emission in frozen solutions is the high degree of heterogeneity in phosphorescence lifetimes. As a typical example, Fig. 2 compares the distribution in lifetime components obtained for azurin in ice and in supercooled water at -12°C. Although in the liquid phase the distribution is so narrow that the decay of phosphorescence is adequately described by a single lifetime, in ice the distribution is wide, indicating that $\tau$ assumes a broad range of values. As the melting temperature of ice is approached, the distribution narrows, and an increasing fraction of the emission has a lifetime as large as that in supercooled liquid. In structural terms, this emission heterogeneity implies that the perturbation of protein structure introduced by the solidification of water is not unique and that the two-phase system provides for a multitude of protein environments, some more stressful than others.

The strain on protein structure produced by freezing is, by and large, promptly relieved by thawing. As shown in Table 1 for azurin, ribonuclease $T_1$, LADH, and AP, $\tau$ of the liquid phase is almost fully restablished, and the freeze–thaw cycle can be repeated several times with only minor variations in spectroscopic properties. For other proteins, such as LDH and GAPDH, which are more subject to irreversible damage of enzymatic activity (Carpenter and Crowe, 1988), the phosphorescence properties are only partly restored.

## Hypothesis on the nature of the ice-induced perturbation

To date, various hypotheses have been formulated concerning the causes of freeze damage and the nature of the underlying perturbation: instability of the folded state at low temperature (subunit dissociation and cold denaturation), the low activity and peculiar properties of interstitial water (drying effect), changes in pH of unfrozen liquid and state of ionization of aminoacid side chains, and finally protein–protein and protein–solute interactions elicited by the large

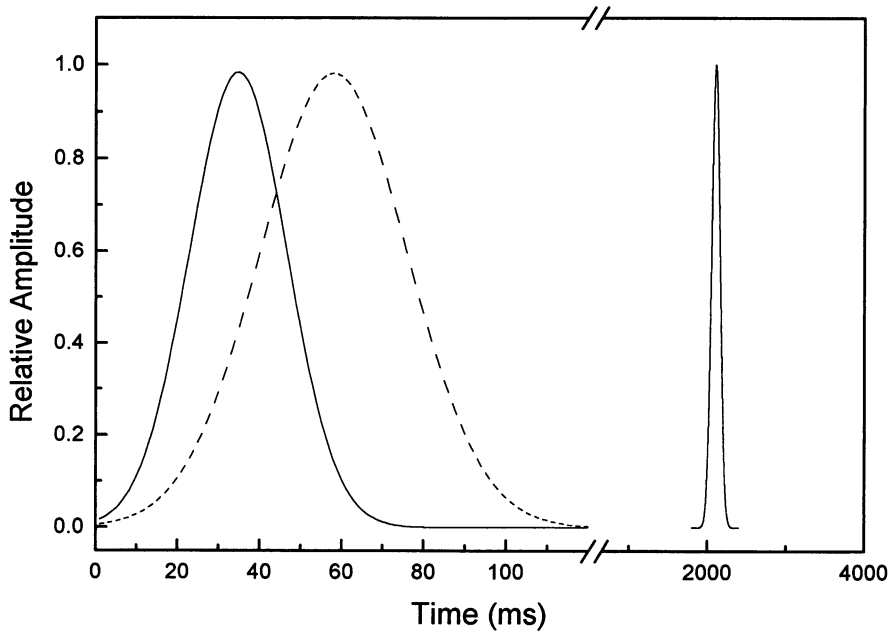


FIGURE 2 Continuous distribution of phosphorescence-lifetime components of azurin in ice and in supercooled solution at -10°C. The dashed curve refers to ice samples that have matured for 10 h at -6°C. Phosphorescence decays were analyzed in terms of a Gaussian distribution of lifetime components by a nonlinear least-squares fitting algorithm implemented by the program Global Analysis (Alcala et al., 1987).

freeze-induced concentration of solutes. To this list we should add protein–ice interactions. For some of the proteins considered in this study all but the last of these possibilities can be discarded. The long lifetimes in supercooled solution, $\tau_0$, prove that low temperature per se is not a destabilizing factor. A drying effect is also readily discounted. The water of hydration crystallizes only in part and at much lower temperature ($T < 140$ K) (Sartor and Mayer, 1994). Further, protein dehydration invariably yields enhanced rigidity, as evidenced in the generally large value of $\tau$ for fully desiccated protein powders (Strambini and Gabellieri, 1984). Similarly, pH effects can also be ruled out. Experiments were conducted in potassium phosphate buffer for its pH stability at subfreezing temperatures (Franks, 1985), but qualitatively similar results were obtained with other buffering salts. Also, between pH 4 and 10 the $\tau$ profile of the protein tested (azurin, LADH, and AP) was largely independent of the starting solution's pH. The interaction among protein molecules normally manifests itself in a concentration dependence of the process. However, variation in protein concentration between 0.1 and 30 $\mu$M (azurin, LADH, and AP) did not affect the lifetime profile, although at even larger concentrations there is a distinct attenuation of the decrease in $\tau$. The same conclusion is reached with respect to possible interactions with buffering salts or NaCl in the unfrozen liquid. Although, as will be shown below, a number of additives do modify the lifetime response to freezing, $\tau_0$ is only moderately sensitive to molar quantities of these solutes.

Finally, we are left to consider protein–ice interactions in some detail. These may take the form of an adsorption process, in the fashion of antifreeze proteins (Chou, 1992) or even of physical distortion of the protein globule confined in the intergranular space of ice crystals by anisotropic compression (Haas et al., 1995). In the first case the protein will be distributed between the liquid and the solid phases and the extent of adsorption will be determined by the volume fraction of liquid water, $V_L$, and the surface area of ice. In the second case the perturbation is expected to be important only when the average size of intergranular spaces is comparable with or smaller than the protein molecular volume. Thus, with respect to $V_L$, adsorption should manifest itself as a gradual process, whereas purely mechanical effects of ice formation should exhibit a more-or-less sharp threshold at some specific $V_L$, a threshold governed by the molecular weight of the protein.

The decrease in phosphorescence lifetime on ice formation, namely, the lifetime ratio $\tau/\tau_0$, was studied as a function of $V_L$. We decreased the size of the liquid-water pool in equilibrium with ice by lowering the temperature and increased it through the addition of NaCl or KCl. The useful temperature range for this test is between 0 and -20°C because, below this limit, the polypeptide rigidity and $\tau$ begin to increase in response to solvent viscosity effects. Moreover, salts will precipitate below their eutectic temperatures and have no further influence on $V_L$. As an example, the effects of temperature and salt concentration on $\tau$ of azurin in ice are displayed in Fig. 3*A*; the pattern is practically identical with those of AP and LADH, the other two proteins examined. The results do emphasize the importance of $V_L$: 1) The reduction of $\tau$ on cooling is very sharp with dilute solutions but becomes less steep the larger the

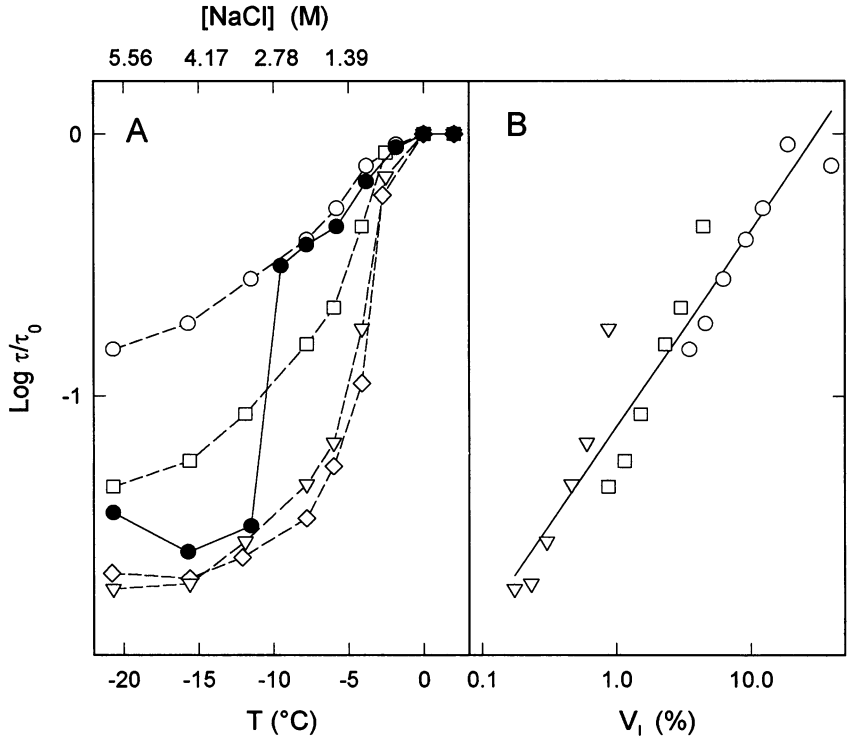


FIGURE 3 (*A*) Phosphorescence lifetime ($\tau/\tau_0 = \tau_{ice}/\tau_{supercooled\ solution}$) versus temperature profiles of azurin in 1-mM Na-phosphate, pH 7 (◇) and in the presence of NaCl or KCl: (▽) 10-mM NaCl, (□) 50-mM NaCl, (○) 200-mM NaCl, and (●) 200-mM KCl. Indicated on the upper $x$ axis is the equilibrium concentration of NaCl in the unfrozen liquid as calculated from the lowering of the freezing-point temperature of an ideal solution ($\Delta T_f = 3.6$ [NaCl]). (*B*) Lifetime data from the three NaCl profiles of (*A*) plotted against the residual fraction of liquid water, $V_L$, in equilibrium with ice calculated assuming the solution to be ideal [$V_L = 3.6$ [NaCl]/(273-$T$)].

initial salt concentration and, hence, $V_L$. At any given temperature, $V_L$ increases and the ratio $\tau/\tau_0$ tends toward unity as more salt is added to the solution, even if the salt concentration in the unfrozen liquid is the same in all samples. 2) There is a clear distinction between the behavior of KCl and NaCl solutions, whose eutectic temperatures are, respectively, -11 and -23°C. Below -11°C KCl crystallizes and ice forms until $V_L$ assumes the same value of dilute solutions. Accordingly, the $\tau$ profile of KCl solutions is practically identical to that of equimolar NaCl solutions above -11°C but shifts abruptly to that of dilute solutions at lower temperatures. 3) The importance of $V_L$ is also depicted in Fig. 3 *B*, where we note that the variation of $\tau$ of azurin is a smooth function of $V_L$, irrespective of the temperature–salt-concentration combination adopted. The lack of a steplike threshold and the finding that substantial changes in $\tau$ occur even with very large volumes of unfrozen liquid ($V_L \sim 10\%$) are two observations that are consistent with the mechanism of protein adsorption to ice and tend to rule out mechanical distortions of the protein globule.

Protein–ice interactions should be influenced also by the texture of ice because, for any $V_L$, the number and size distribution of ice crystals will determine the total ice surface area and the actual size of intergranular spaces. The texture of ice depends, among other factors, on the cooling rate and on aging; slow cooling and prolonged maturing promote the formation of few and large ice crystals (Franks, 1985). Protein solutions seeded with a small ice crystal at -2°C and then cooled at rates of ~200 or 1°C $min^{-1}$ exhibit substantially different lifetime responses. The results indicate that slow cooling yields a smaller perturbation of $\tau$. At the slower cooling rate, the ratio $\tau/\tau_0$, at -6°C, is greater by 20% with azurin, 46% with AP, and 105% with LADH. Likewise, rapidly cooled samples left to anneal for 10 h at -6°C showed a recovery in the ratio $\tau/\tau_0$, with increases of 40% with AP, 70% with azurin, and 2.5 folds with LADH. In either test, rate of cooling or annealing, a smaller perturbation of protein structure is associated with the reduction in ice surface area, again a correlation between $\tau$ and ice morphology that is consistent with decreased protein adsorption.

## Effects of cryoprotectants

The above findings indicate that the reasons for protein destabilization in the frozen state must be sought in the direct interaction of the polypeptide with the solid phase. To verify whether there is a direct link between the loosening of the native structure revealed by $\tau$ and the onset of degenerative processes that lead to enzyme inactivation–denaturation we tested the influence of cryoprotectants on the lifetime response to freezing. Among these, of particular interest are those substances, such as polyols and sugars, that even in moderate concentrations afford a large degree of protection against freeze damage (Carpenter and Crowe, 1988). Fig. 4 shows the influence of glycerol and sucrose, compounds commonly employed as cryoprotectants, on the ratio $\tau/\tau_0$ of azurin, LADH, and AP at -10°C. The results are compared with those obtained with equimolar solutions of "neutral" salts such as NaCl and K-phosphate that presumably account mainly for the colligative effects ($V_L$) of the solutes. Although in liquid solutions molar concentrations of these compounds barely affect the internal flexibility of these proteins ($\tau_0$), in partly frozen solutions even moderate quantities display a remarkable ability to attenuate the reduction in $\tau$. The effect is more dramatic with sucrose, which is also a more potent cryoprotectant, and with azurin and LADH, proteins that compared with AP undergo larger $\tau$ perturbations on freezing. This correlation between the

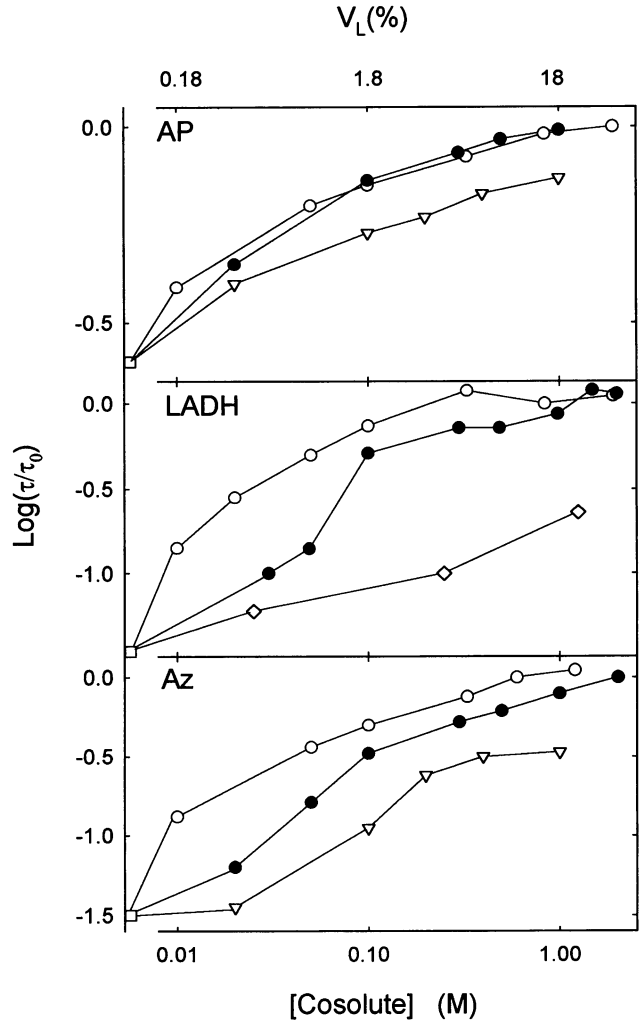


FIGURE 4 Effects of cosolutes on the lifetime ratio $\tau/\tau_0$ of some proteins at -10°C. The solutes are (○) sucrose, (●) glycerol, (▽) NaCl, and (◇) Na-phosphate. All proteins were in 1-mM Na-phosphate buffer, pH 7. Inasmuch as chloride ions bind weakly to LADH and lower the value of $\tau_0$, NaCl was replaced by Na-phosphate. The protein concentration ranged between 3 and 5 $\mu$M, and ice was formed from a supercooled solution at -5°C seeded with a small ice crystal. The data shown are the averages of two independent measurements. Generally, the standard error in $\tau$ did not exceed 10%.

attenuation of the ice-induced unfolding transition and the cryoprotective potential of the solute suggests a close connection between the structural alterations revealed by the triplet probe and those implicated in freeze damage.

In conclusion, by means of the intrinsic phosphorescence emission it was possible to demonstrate for the first time that the solidification of water alters the native fold of proteins deep down to the inner core of the macromolecule, the generalized increase in flexibility reflecting an extensive unfolding of the polypeptide. The results reported here suggest that the dominant perturbation originates from a direct interaction of the macromolecule with ice. Recent structural studies of protein adsorbed on various substrates such as metal oxides, silica, and charged polystyrene (Haynes and Norde, 1995) have demonstrated a considerable loss of secondary structure and a profound destabilization with respect to thermal unfolding. Because the variation in $\tau$ on freezing is consistent with structural alterations of this kind, and the tests presented here tend to exclude other hypotheses, we propose that the strain on the native protein fold in frozen solutions is to be attributed to the adsorption of the macromolecule onto the surface of ice. In this new perspective the stabilizing action of cosolutes should then be regarded as a combined effect of 1) lowering the freezing temperature, i.e., increasing $V_L$ (colligative properties), 2) decreasing the adsorption affinity to a surface partly coated by cosolutes, and 3) opposing adsorption-induced structural deformations (preferential hydration). Systematic studies can now be undertaken to assess the relative contributions of these factors.

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